Supplemental Data

Committed Neuronal Precursors Confer

Astrocytic Potential on Residual

Neural Precursor Cells

Masakazu Namihira, Jun Kohyama, Katsunori Semi, Tsukasa Sanosaka, Benjamin Deneen, Tetsuya Taga, and Kinichi Nakashima

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde and processed for immunostaining as described (Takizawa et al., 2001). Cells were stained with one of the following primary antibodies: rabbit anti-GFP (1:500, Molecular Probe), mouse anti-β III-Tubulin (Tuj1; 1:500, Sigma), mouse anti-GFAP (1:500, Sigma). Secondary antibodies were Alexa488-conjugated donkey anti-rabbit IgG (1:500, Molecular Probe) or Cy3-conjugated donkey anti-mouse IgG (1:500, Chemicon). Nuclei were stained using bisbenzimide H33258 fluorochrome trihydrochloride (Nacalai Tesque). All experiments were independently replicated at least three times.

Immunohistochemistry

Embryos were fixed in 4% paraformaldehyde and their brains were sectioned at 6 or 12 μm with a cryostat. For staining with anti-cleaved Notch and anti-DLL1 antibodies, antigen retrieval was accomplished by autoclaving sections for 15 minutes at 105˚C in 10 mM sodium citrate buffer (pH 6.0) and processing for immunostaining as described (Tokunaga et al., 2004; Yoshimatsu et al., 2006). Antibodies used were anti-DLL1 (1:100, Santa Cruz, H265), -JAG1 (1:100, Santa Cruz, H114), -cleaved Notch (1:100, Cell Signaling), -NGN1 (1:100, Santa Cruz, A20), -NFIA (1:1000, Active-Motif), -Nestin (1:200, Rat-401, Chemicon), and -Tbr2 (1:500, Chemicon). Stained sections were visualized with a confocal microscope (LSM510, Zeiss) or fluorescence microscope (Zeiss Axiovert 200M, Zeiss, or Olympus BX50, Olympus).

RT-PCR

Total RNA was extracted from cells expressing GFP alone, or GFP together with NICD or NFIA, using Isogen (Nippon Gene) according to the manufacturer's instructions. Reverse transcription reactions were carried out using the SuperScriptII First Strand Synthesis System for RT-PCR (Invitrogen) according to the kit protocol. Primer sequences are available upon request.

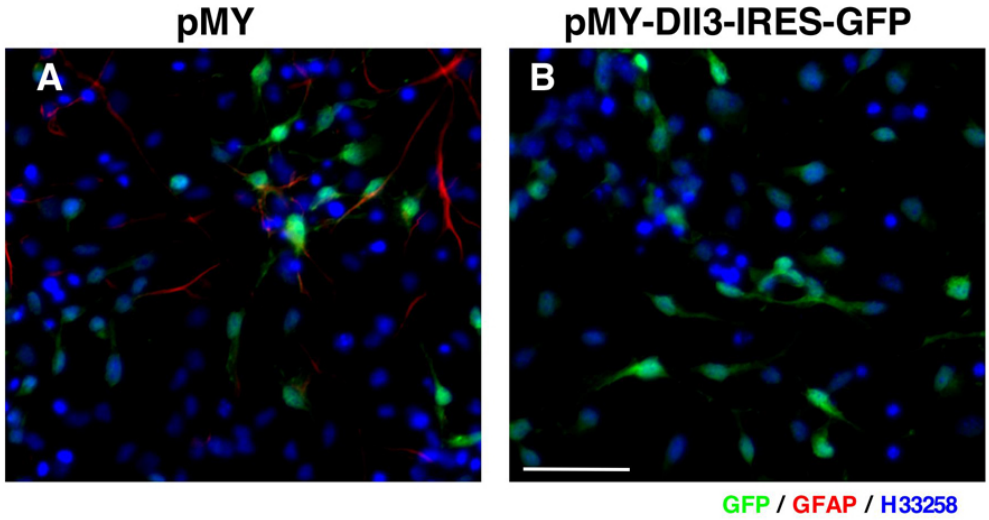

Figure S1. Notch Signal inhibitor Dll3 in E11.5 NPCs Suppresses Astrocytic Differentiation in Co-Culture Conditions
E11.5 NPCs infected with retroviruses engineered to express GFP alone (A) or GFP together with Dll3 (B) were co-cultured with embryonic cortical neurons in the presence of LIF for 4 days. The cells were stained with antibodies against GFP (green) and GFAP (red), and with H33258 to identify nuclei (blue). Scale bar = 20 μm.

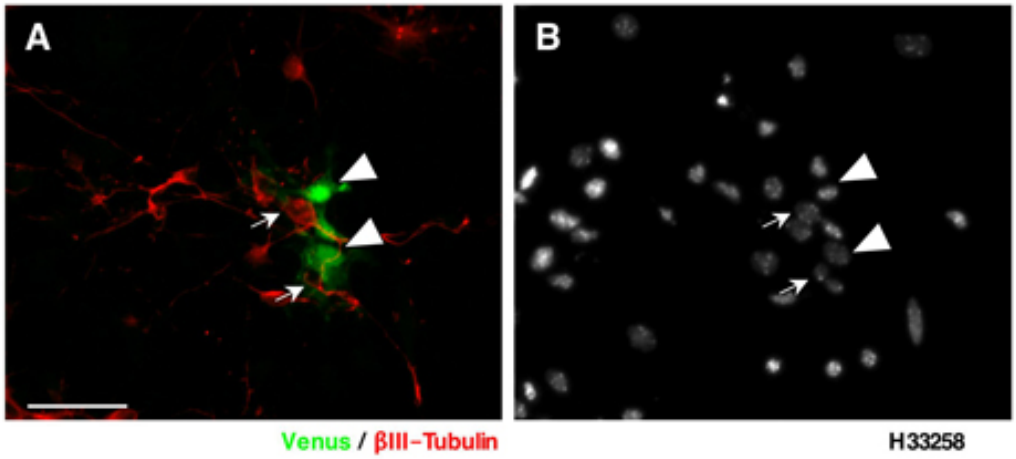

Figure S2 Notch Signaling Is Activated in NPCs Located in Close Contact with Embryonic Cortical Neurons
(A) E11.5 NPCs were transfected with TP1-Venus Notch-activation reporter plasmid and co-cultured with embryonic cortical neurons for 4 days. The cells were stained with antibodies against GFP (green, arrowheads) and Tuj1 (red, arrows).
(B) H33258 staining indicates nuclei of cells in (A). Arrowheads and arrows in (A) and (B) indicate representatives of Notch-activated cells and immature neurons, respectively.
Scale bar = 20 μm.

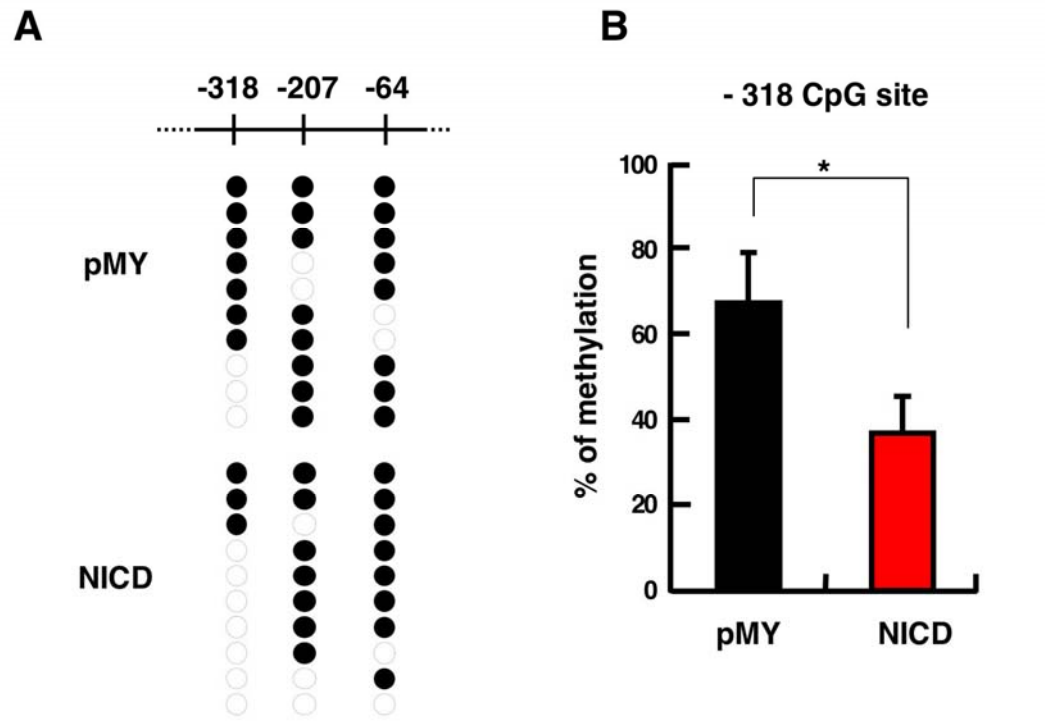

Figure S3. A CpG Site within *S100β* promoter Is Demethylated by expression of NICD in NPCs

(A) E11.5 NPCs were infected with GFP control (pMY) and GFP-NICD-expressing retroviruses, and were cultured for 4 days with bFGF. After cell-sorting based on GFP fluorescence, genomic DNA was extracted from the cells and the methylation status of three CpG sites within the *S100β* gene promoter was examined by bisulfite sequencing. Closed and open circles indicate methylated and unmethylated CpG sites, respectively.

(B) Methylation frequency of the CpG site at position -318 in the *S100β* gene promoter. Mean ± S.D. (N = 3). Statistical significance was evaluated by the Student t-test. *, $p < 0.05$.

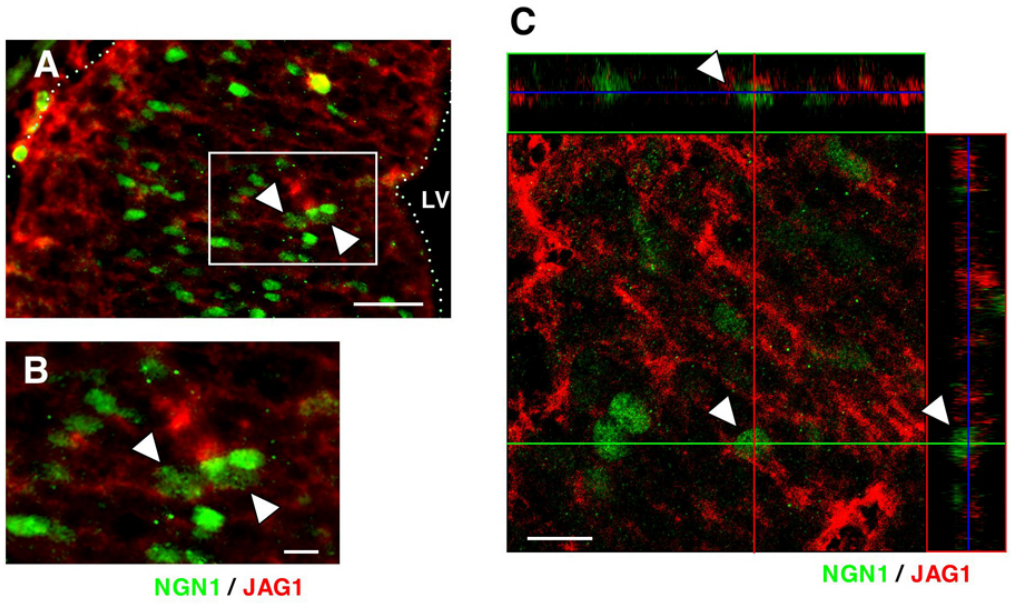

Figure S4. JAG1 Is Expressed in NGN1-Expressing Cells

(A) E11.5 forebrain sections (6 μm) were stained with antibodies against NGN1 (green) and JAG1 (red). JAG1 was expressed in NGN1-positive differentiating neurons (arrowheads in **A**-**C** mark representatives). Scale bar = 20 μm.

(B) High-magnification view of the boxed area in (**A**). Scale bar = 10 μm. **C**, Co-expression of JAG1 and NGN1 in these cells was confirmed by three-dimensional digital imaging of a brain section immunostained as in (**A**). Scale bar = 10 μm.

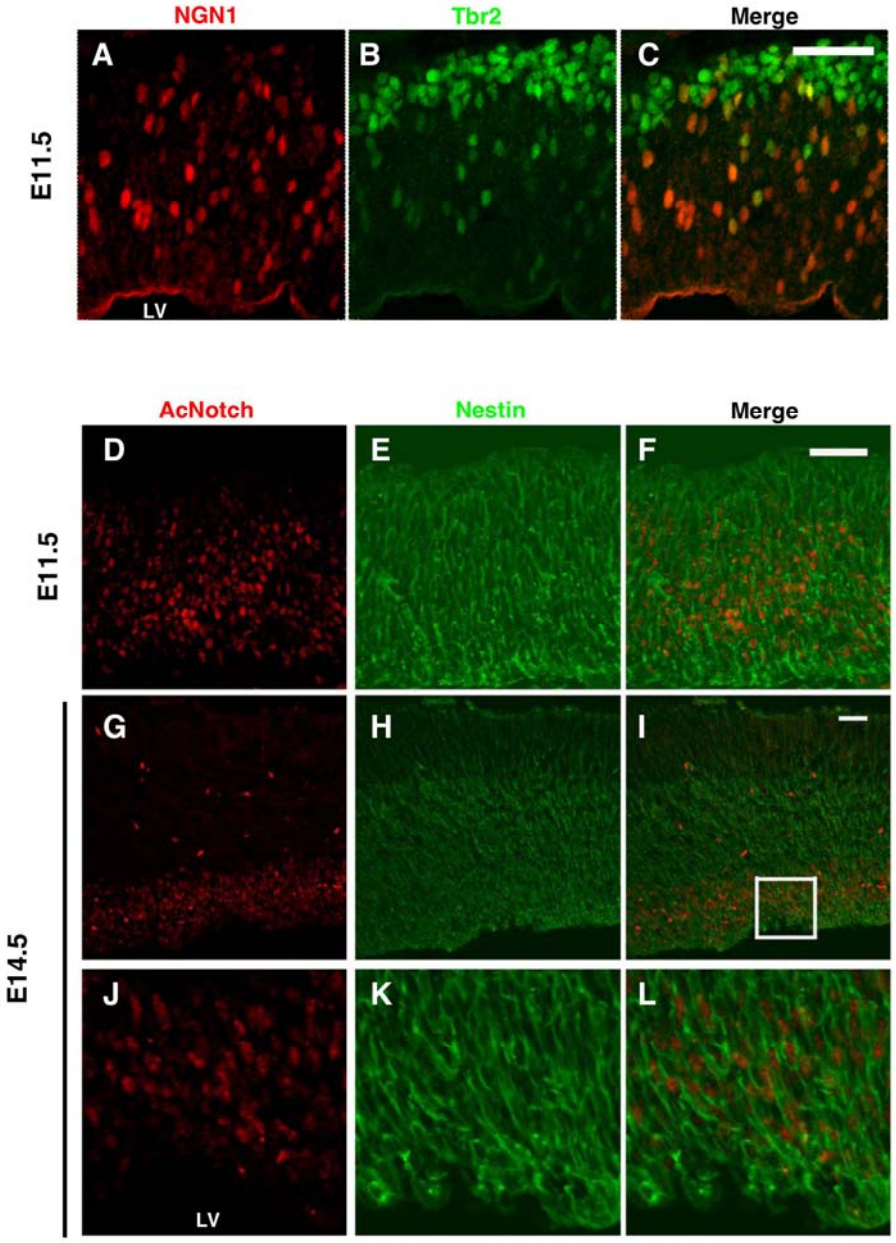

Figure S5. Characterization of NGN1-Expressing Cells and Notch-Activated NPCs
(A–C) E11.5 forebrain sections (6 μm) were stained with antibodies against NGN1 (red) and Tbr2 (green). Tbr2 was expressed in NGN1-positive committed neuronal precursors. Scale bar = 50 μm.
(D–I) E11.5 (D-F) and E14.5 (G-I) forebrain sections (6 μm) were stained with antibodies against AcNotch (red) and Nestin (green).
(J–L) High-magnification view of the boxed area in (I). Scale bar = 50 μm.

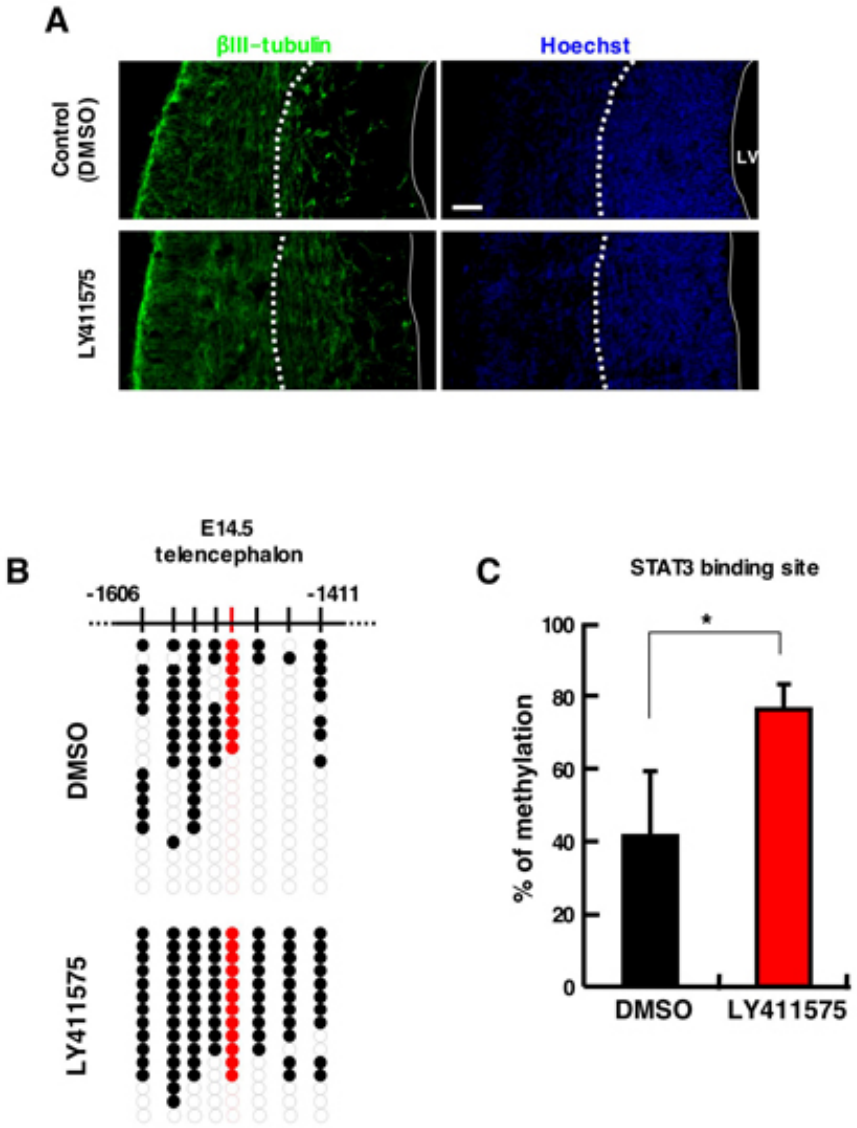

Figure S6. **Suppression of Notch Activation by LY411575 Treatment In Vivo Leads to an Overproduction of Neurons and Inhibition of the *gfap* Promoter Demethylation in NPCs**
(A) E14.5 forebrain sections of DMSO- (upper panels) or LY411575-treated (lower panels) mice were stained with antibodies against β-III Tubulin (left panels, green). Hoechst staining indicates nuclei (right panels, blue). The white dotted line marks the boundary between the intermediate zone and VZ/SVZ in the telencephalon. LV, lateral ventricle. Scale bar = 50 μm. (B) Bisulfite sequencing results for the CpG site within the STAT3 recognition sequence (red), and other CpG sites around this sequence of the *gfap* promoter, in NPCs purified from telencephalon of DMSO- or LY411575-treated SOX2-GFP transgenic mice at E14.5. C, Methylation frequency of the CpG site within the STAT3-binding sequence in the *gfap* promoter. Mean ± S.D. (N = 3). Statistical significance was examined by the Student t-test. *$p < 0.05$.

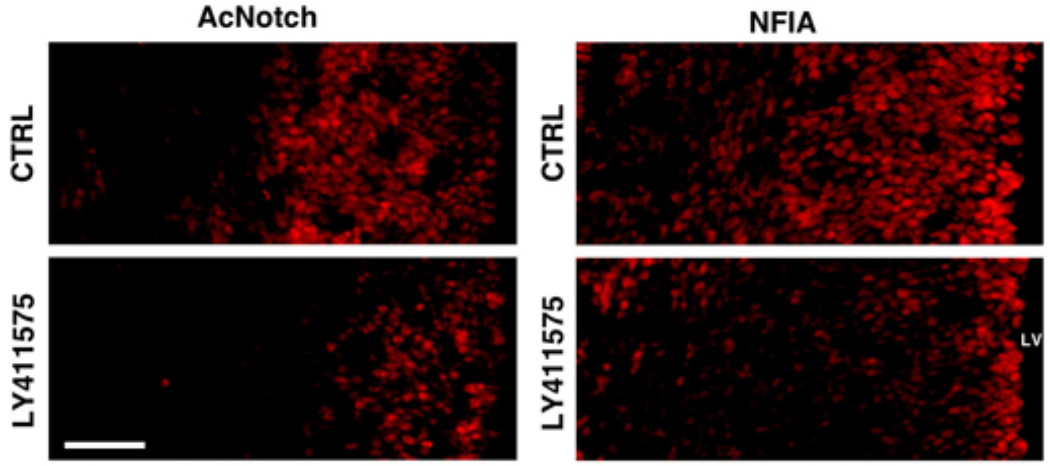

Figure S7. The Number of NFIA-Positive Cells in VZ Was Significantly Reduced in LY411575-Treated Embryos

E14.5 forebrain sections (12 μm) of DMSO- (upper panels) or LY411575-treated (lower panels) mice were stained with antibodies against cleaved Notch (AcNotch, left panels) or NFIA (NFIA, right panels). The area occupied by NFIA-positive cells in the VZ was significantly reduced in LY411575-treated embryos. LV, lateral ventricle. Scale bar = 50 μm.

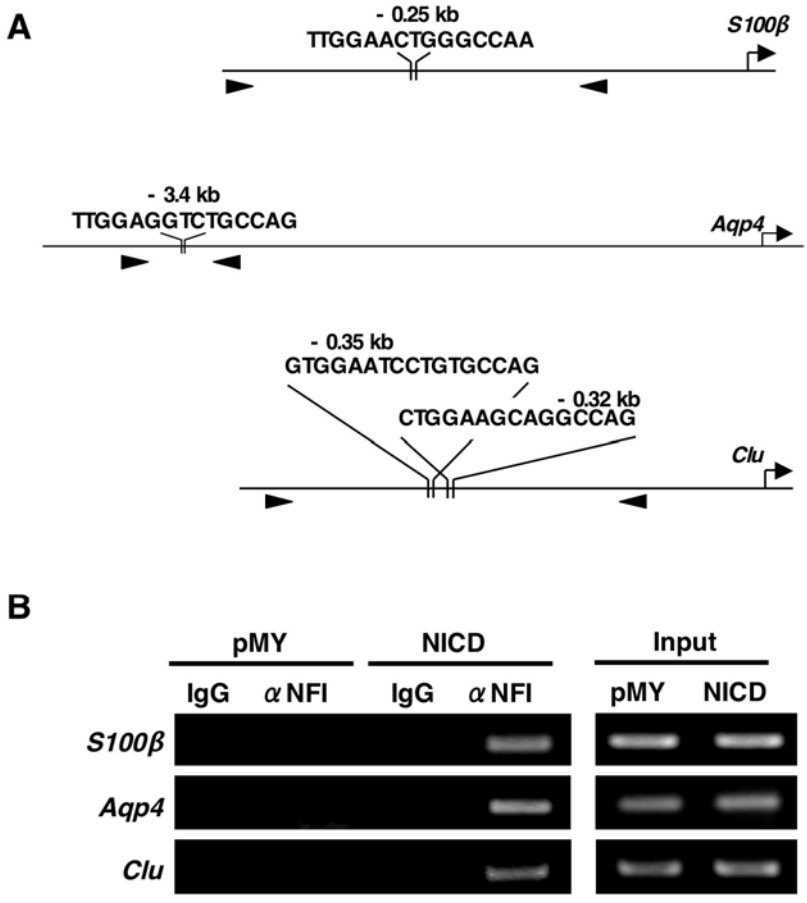

Figure S8. NFI-Binding to Promoter Regions of astrocyte-Specific genes in NICD-Expressing NPCs

(A) Schematic diagram of putative NFI-binding sequences located upstream of the transcription initiation sites (arrows) of mouse *S100β*, *aquaporin 4* (*Aqp4*) and *clusterin* (*Clu*). Arrowheads indicate the locations of primers used in PCR for ChIP samples in (B).

(B) ChIP assay with anti-NFI antibody for GFP- and GFP-NICD-expressing retrovirus-infected NPCs cultured as in Fig. 1G. Binding of NFIA to the *S100β*, *aquaporin 4* (*Aqp4*) and *clusterin* (*Clu*) promoters was observed in response to NICD expression in E11.5 NPCs.

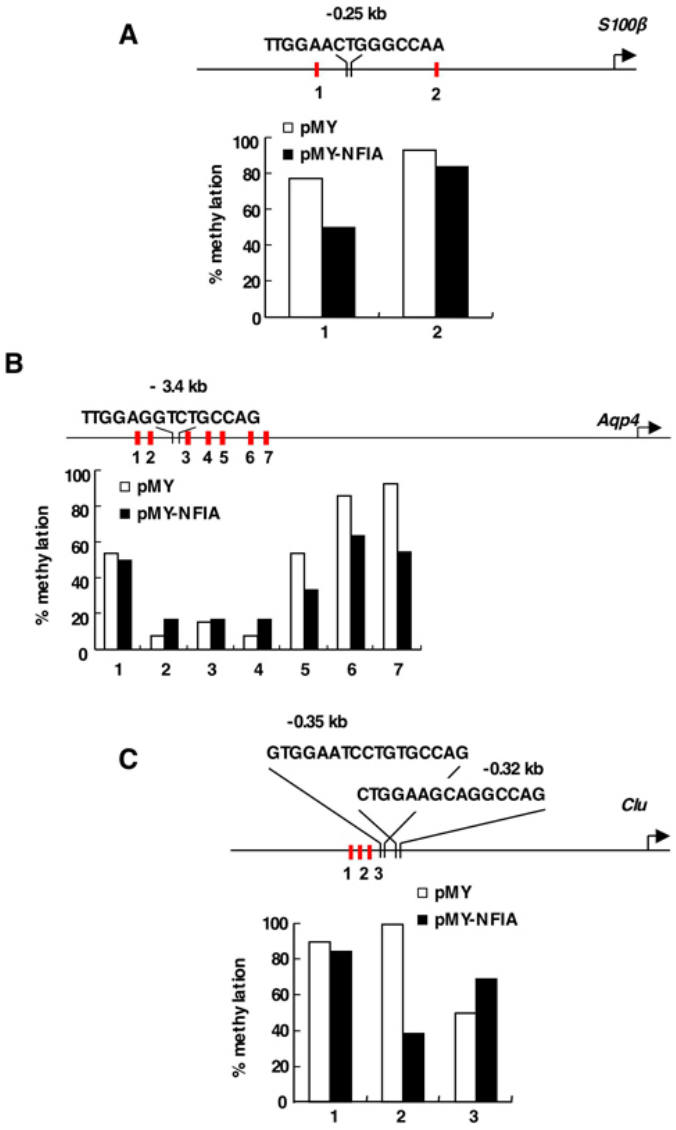

Figure S9. Demethylation of Particular CpG Sites in the Astrocytic Gene Promoters Was Induced in NFIA-expressing NPCs

Bisulfite sequencing of mouse *S100β* (A), *aquaporin 4* (*Aqp4*, B) and *clusterin* (*Clu*, C) promoters in GFP-expressing (pMY) or GFP- and NFIA-expressing (pMY-NFIA) E11.5 NPCs. CpG sites near the putative NFI-binding sequence(s) in each promoter are indicated as red boxes. PCR products amplified from bisulfite-treated genomic DNA were cloned into the T7Blue vector and sequenced. Methylation frequencies in PCR clones at the indicated CpG sites of each promoter are expressed as a percentage of the total number of clones sequenced (10 to 15 clones).

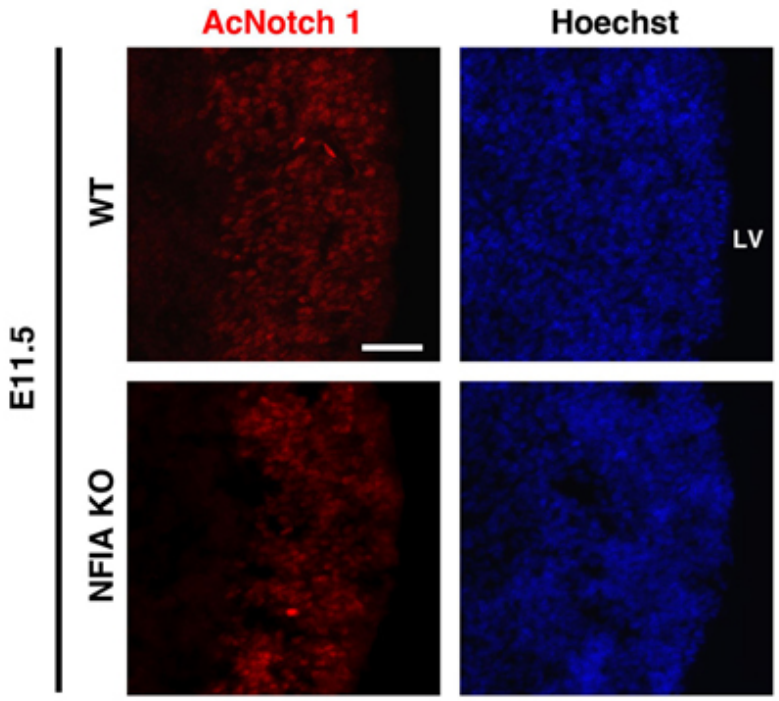

Figure S10. Notch Signaling Is Activated in the NFIA-Deficient Brain
E11.5 forebrain sections of WT (upper panels) and NFIA-KO (lower panels) mice were stained with antibodies against activated Notch (AcNotch in left panels, red). Hoechst staining indicates nuclei (right panels, blue). LV, lateral ventricle. Scale bar = 50 μm.